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Ottawa Hull K1A 0C9

(21) (A1) 2,110,543
(22) 1993/12/02
(43) 1994/06/10

(51) INTL.CL.[5] C12N-009/00; C07K-015/28; C07K-015/26; C07K-015/12; C08F-008/30; C08B-015/06; C08B-030/18; C08B-037/00; A61K-047/48; A61K-039/00; A61K-037/24; A61K-037/66

(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

# (54) Peg Hydrazone and Peg Oxime Linkage Forming Reagents and Protein Derivatives Thereof

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(30) (US) 07/987,739 1992/12/09

(57) 102 Claims

5,091,3/93

ABSTRACT

The present invention provides methods and compounds for modifying polypeptides with PEG or other water-soluble organic polymers. Novel water-soluble polymer reagents are provided for.

The water-soluble polymer reagents of the subject invention include hydrazine, hydrazine carboxylate, semicarbazide, thiosemicarbazide, carbonic acid dihydrazide, carbazide, thiocarbazide, and arylhydrazide derivatives as well as oxylamine derivatives of water-soluble organic polymers, such as polyethylene glycol, polypropylene glycol, polyoxyethylated polyol, heparin, heparin fragments, dextran, polysaccharides, polyamino acids, and polyvinyl alcohol.

Also provided for, are polypeptides of interest derivatized by the subject water-soluble polymer reagents.

Kits for modifying polypeptides with the subject water-soluble polymer reagents are also provided.

Field of the Invention

This invention relates to water-soluble polymers, such as monomethoxypoly(ethylene glycol), that are modified to form a hydrazone linkage with an aldehyde group on a protein, and the invention also relates to protein molecules modified by these water-soluble polymers.

This invention further relates to such water-soluble polymers that are modified to form an oxime linkage, and protein molecules modified thereby.

Background Art

Protein and other similar organic molecules may be chemically modified by covalent conjugation to water-soluble organic polymers such as polyethylene glycol (PEG) The production of such protein conjugates is of interest because of the desirable properties conferred on polypeptides by the attachment of the water-soluble polymers. These desirable properties include increased solubility in aqueous solutions, increased stability during storage, reduced immunogenicity, increased resistance

to enzymatic degradation, compatibility with a wider variety of drug administration systems, and increased in vivo half-life. These properties that are brought about by the derivatization of polypeptides with PEG or other water-soluble polymers are especially of interest when the polypeptide is to be used as a therapeutic agent injected into the body or when the polypeptide is to be used in assays, usually immunoassays, for the detection and/or quantification of a compound of interest.

The attachment of reporter groups, ligands, etc. to proteins through a glycoprotein's carbohydrate moiety has been described [Weber, P. and Hof, L. (1975) Biochem. Biophys. Res. Commun. 65, 1298-1302; O'Shannessy, D.J., Dobersen, M.J., and Quarles, R.H. (1984) Immunol. Lett. 8, 273-277; O'Shannessy, D.J. and Quarles, R.H. (1985) J. Appl. Biochem. 7, 347-355; Chua, M.-M., Fan, S.-T., and Karush, F. (1984) Biochim. Biophys. Acta 800, 291-300; O'Shannessy, D.J. and Wilchek, M. (1990) Anal. Biochem. 191, 1-8; Koppel, G.A. (1990) Bioconjugate Chem. 1, 13-23]. A number of groups have been covalently attached in this manner including biotin, fluorescent probes, anticancer compounds, and solid supports.

U.S. Patent No. 4,847,325 describes the possibility of synthesizing a PEG-amine, PEG-hydrazide or PEG-hydrazine and attaching it to a glycoprotein. However, no experimental evidence was given that these PEG-derivatives had been synthesized, that these PEG-derivatives could modify an oxidized glycoprotein, and what the resulting biological properties of these putative PEG-proteins might be.

The publication by Kogan, T.P., Synthetic Communications, 22(16), 2417-2424 (1992), describes the synthesis of a monomethoxypoly(ethylene glycol)-hydrazide.

The enzyme peroxidase has been modified with PEG through its carbohydrate groups [Urrutigoity, M. and Souppe, J. (1989) Biocatalysis 2, 145-149]. In this modification PEG-diamine was reacted with oxidized peroxidase, and the resulting imine was reduced with borohydride to form a stable bond between PEG and the carbohydrate group on the protein. Three molecules of PEG-20,000 were attached to the enzyme. A possible problem in using PEG-diamine in this manner is intermolecular cross-linking taking place between the protein molecules with PEG diamine functioning as the cross-linker. Another drawback of using PEG-diamine is the consumption of two available aldehyde groups for each PEG-diamine attached to the protein, thus lowering the potential number of sites for PEG incorporation.

Besides the hydrazone forming mPEG derivatives, also synthesized is a series of oxime forming mPEG derivatives. Oximes are formed by the reaction of hydroxylamine or oxylamine derivatives with aldehyde or ketone groups. Polystyrene substituted benzophenone oximes have been used as supports for solid-phase peptide synthesis [DeGrado, W.F., and Kaiser, E.T. (1980) J. Org. Chem. 45, 1295-1300]. In this example the growing peptide chain is coupled to the oxime group via an ester linkage. The substituted oxime bond is quite stable, and even unsubstituted aldoximes show good stability towards the Beckmann rearrangement requiring 60 hr at 100° in the presence of silica gel to yield the reaction

[March, J. (1985) Advanced Organic Chemistry, New York-John Wiley & Sons, pp 987-989]. The oxime linkage has been used to couple morpholinodoxorubicin to an antibody [Mueller, B.M., Wrasidlo, W.A., and Reisfeld, R.A. (1990) Bioconjugate Chem. 1, 325-330]. In this example the ketone group of morpholinodoxorubicin was reacted with aminooxyacetic acid. The newly coupled free acid group was activated, and morpholinodoxorubicin was linked to the free amino groups of lysine on a monoclonal antibody.

A number of proteins have been modified by PEG. For a review see Inada, Y., Yoshimoto, T. Matsushima, A., and Saito, Y. (1986) Trends Biotechnol. 4: 68-73. A number of patents have issued and applications published in this field as listed below: U.S. Pat. No. 4,179,337; U.S. Pat. No. 4,609,546; U.S. Pat. No. 4,261,973; U.S. Pat. No. 4,055,635; U.S. Pat. No. 3,960,830; U.S. Pat. No. 4,415,665; U.S. Pat. No. 4,412,989; U.S. Pat. No. 4,002,531; U.S. Pat. No. 4,414,147; U.S. Pat. No. 3,788,948; U.S. Pat. No. 4,732,863; U.S. Pat. No. 4,745,180; EP No. 152,847; EP No. 98,110 published January 11, 1984. The above patents and patent publications also describe the use of other water-soluble polymer protein modifying reagents including but not restricted to polypropylene glycol (PPG), polyoxyethylated polyol (POP), heparin, heparin fragments, dextran, polysaccharides, polyamino acids including proline, polyvinyl alcohol (PVA) and other water-soluble organic polymers.

A recent patent publication (WO90/12874) describes the preparation of an mPEG-EPO in which the EPO contains a cysteine residue introduced by genetic

engineering. A cysteine specific mPEG-reagent is then covalently attached to the genetically engineered free sulfhydryl group. Only one mPEG molecule could be incorporated into EPO and no evidence of this incorporation was presented. Also no biological or biophysical properties of the resulting mPEG-EPO were described.

Erythropoietin is a glycoprotein which regulates red blood cell production. Erythropoietin exerts its biological effect by binding to receptors on erythroid precursors (Krantz, S.B., Blood 77: 419-434 (1991)). The binding of erythropoietin to its receptor causes erythroid precursors to proliferate and differentiate into mature red blood cells. Other growth factors such as interleukin 3 or granulocyte-macrophage colony-stimulating factor also are involved in erythropoiesis along with cofactors such as iron, folic acid, and vitamin B12. Currently erythropoietin is approved for use in anemia of chronic renal failure in both dialysis and predialysis patients and for the anemia of HIV infection and in combination with zidovudine therapy. Current uses for erythropoietin under study include anemia of cancer, presurgical autologous blood donation, and perisurgical adjuvant therapy.

Erythropoietin consists of 165 amino acids which includes two disulfide bridges. Erythropoietin has four carbohydrate chains emanating from the protein backbone. Three of the carbohydrate groups are N-linked and are attached to asparagines 24, 38, and 83. Also there is one O-linked carbohydrate group secured to serine 126. The carbohydrate chains are branched and consist of fucose, galactose, N-acetylgalactosamine, N-acetylglucosamine, mannose,

and sialic acid. The carbohydrate composition of erythropoietin is heterogeneous as determined by Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987) J. Biol. Chem. 262, 12059-12076. Carbohydrate groups account for about 40% of the protein's weight. The carbohydrate groups on erythropoietin are believed to increase the solubility of erythropoietin and prolong its serum half-life.

Several limitations exist with respect to which polypeptides may be covalently conjugated to water-soluble polymers and the extent to which the polypeptides can be modified. Different water-soluble polymer reagents vary with respect to the functional groups that provide for coupling to amino acid residues in polypeptides of interest. Specific functional groups provide for the coupling of water-soluble polymers to specific amino acid residues.

Modification of lysine residues using different mPEG-reagents having different properties such as succinimidyl carbonate-PEG, succinimidyl succinate-PEG, imidate-PEG, cyanuric chloride-PEG, carbonyldiimidazole-PEG, and PEG-phenylcarbonate derivatives (4-nitrophenol and 2,4,5-trichlorophenol) have been described. Each reagent has its own specific property. The subject application involves new carbohydrate PEG modifying agents with different specificities to oxidized carbohydrate groups analogous to the different lysine modifying PEG-derivatives.

Glycoproteins, i.e., polypeptides covalently joined to a carbohydrate molecule or molecules, provide additional opportunities for providing different methods of water-soluble polymer derivatization of a polypeptide because of the

presence of the carbohydrate moieties on the polypeptide. Water-soluble polymer reagents may be coupled directly to the carbohydrate moieties of glycoproteins as opposed to the amino acid polypeptide backbone, i.e., various functional groups present on the polypeptide, of the glycoprotein. It may be advantageous to couple water-soluble reagents to the carbohydrate moiety of a glycoprotein rather than to the polypeptide backbone amino acids because of differences in charge displacement, steric hinderance, amino acid residues at active sites, and other problems that may disrupt the structure and function of the polypeptide component of the water-soluble polymer modified glycoprotein.

By providing for water-soluble polymer reagents that may be coupled to the carbohydrate moiety of glycoproteins it may be possible to covalently conjugate water-soluble polymers to proteins without substantially adversely affecting the biological activity of proteins that would be adversely affected through coupling at other amino acid residues.

## SUMMARY OF THE INVENTION

The present invention provides methods and compositions for modifying polypeptides with derivatives of water-soluble organic polymers, i.e., water-soluble polymer reagents, that form a hydrazone linkage with an aldehyde group or group with similar chemical reactivity, e.g., ketones, lactols, activated carboxylic acids or activated carboxylic acid derivatives on a polypeptide. Novel hydrazide, semicarbazide, aryl hydrazide, thiosemicarbazide, hydrazide carboxylate, carbonic acid dihydrazide, carbazide, and thiocarbazide derivatives of

polyethylene glycol (PEG) and other water-soluble polymers are provided. One or more of the water-soluble polymer reagents may be coupled to individual polypeptides or similar organic molecules to form hydrazones that link the polypeptide to water-soluble polymers.

Another aspect of the subject invention is to provide for proteins, particularly glycoproteins, modified by the covalent attachment of hydrazone linkage water-soluble polymer derivatives.

Also disclosed are methods and compositions for modifying polypeptides with derivatives of water-soluble organic polymers that form an oxime linkage with the above-mentioned aldehyde or similarly reactive groups. Novel oxylamine derivatives as listed hereinbelow, of polyethylene glycol (PEG) and other water-soluble polymers are provided and wherein one or more of the water-soluble polymer reagents may be coupled to individual polypeptides or similar organic molecules to form oximes that link the polypeptide to water-soluble polymers.

Another aspect of the subject invention is to provide for proteins, particularly glycoproteins, modified by the covalent attachment of oxylamine water-soluble polymer derivatives.

The water-soluble polymer reagents of the subject invention include hydrazone linkage and oxime linkage forming derivatives of polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group, polyoxyethylated polyols, polyvinyl alcohol, polysaccharides, polyvinyl ethyl ethers, and $\alpha,\beta$-

Poly[(2-hydroxyethyl)-DL-aspartamide] and other water-soluble organic polymers. Polyethylene glycol water-soluble polymers include polyethylene glycol where one of the terminal hydroxyl group is modified with an R group, i.e., RO-PEG, where R may be alkyl, aryl, alkyaryl, aroyl, alkanoyl, benzoyl, arylalkylethers, cycloalkyl, cycloalkylaryl, and the like. The water-soluble polymers listed are only exemplary of water-soluble polymers represented by P. Various derivatives of the specifically recited water-soluble polymers are also contemplated, provided that the derivatives are water-soluble. More preferably, the water-soluble polymer P is selected from the group consisting of polyethylene glycol and derivatives thereof, the monomethyl ether of polyethylene glycol (mPEG) being particularly preferred (so as to avoid cross-linking between proteins).

Polypeptides of interest for water-soluble polymer derivatization by the subject water-soluble polymer include hormones, lymphokines, cytokines, growth factors, enzymes, vaccine antigens, and antibodies. Water-soluble polymer derivatization of erythropoietin (EPO), especially recombinant erythropoietin, and precursors, intermediates and mimetics thereof, are of particular interest.

Another aspect of the invention is to provide erythropoietin that has been partially oxidized and subsequently combined with (i) a semicarbazide derivative of the monomethoxypoly (ethylene glycol) (mPEG), so as to produce mPEG derivatized erythropoietin molecules containing 17-25 mPEG molecules/molecule of erythropoietin (joined through hydrazone linkages), (ii) a carboxylate hydrazide

derivative of mPEG so as to produce derivatized EPO containing about 22-32 mPEGs/EPO (joined through hydrazone linkages), and (iii) oxylamine derivatives of mPEG so as to produce derivatized EPO containing about 3-36 mPEGs/EPO (joined through oxime linkages), all as measured by gel filtration retention time.

Another aspect of the invention is to provide methods of activating polypeptides for covalent conjugation with the subject water-soluble polymer reagents.

## DESCRIPTION OF THE DRAWINGS

Figure 1a shows an HPLC chromatogram of EPO. Figure 1b shows an HPLC chromatogram of EPO modified with a hydrazine derivative of mPEG5000. Figure 1c shows an HPLC chromatogram of EPO modified with a succinimide ester of mPEG5000.

Figure 2 is a graph of the hematocrit level of mice treated with mPEG5000-EPO containing different amounts of attached mPEG (28, 18 and 12 mPEGs/molecule of EPO). The EPO derivatized with 18 or 28 mPEG5000/molecule are derivatized using the subject semicarbazide compound. EPO derivatized with 12 mPEG5000/molecule is derivatized using the subject hydrazide compound.

Figure 3 is a graph showing the ability of EPO, hydrazide mPEG5000 EPO (12 PEG/EPO), hydrazide mPEG12000-EPO (6 PEG/EPO), thiosemicarbazide mPEG5000-EPO (25 PEG/EPO), semicarbazide mPEG12000-EPO (14 PEG/EPO), and semicarbazide mPEG12000-EPO (29 PEG/EPO) to bind a monoclonal antibody specific for EPO in an ELISA assay.

Figure 4 is a graph showing a comparison of the biological activity of EPO when modified with either

mPEG-Hydrazide (HY) or mPEG-Semicarbazide (SC). Two different molecular weights (8500 and 5000) of mPEG were used in the comparison. Mouse albumin is used as the control.

Figure 5 is a plot showing the hematocrit level of mice treated with mPEG8500-EPO containing different amounts of attached mPEG (34, 20 and 12 mPEGs). EPO derivatized with 34 or 20 mPEG85000/molecule are derivatized using the subject semicarbazide compound. EPO derivatized with 12 mPEG85000/molecule is derivatized using the subject hydrazide compound. Mouse albumin is used as the control.

Figure 6 is a graph showing the results of ELISA assays for mPEG modified EPO using a Clinigen® EPO EIA test kit. In the legend, SC5-24 refers to semicarbazide mPEG5000 modified EPO with 24 molecules of mPEG/molecule of EPO, SC5-18 refers to the semicarbazide mPEG5000 modified EPO with 18 molecules of mPEG semicarbazide /molecule of EPO.

Figure 7 is a graph showing the circulating half-life of EPO in plasma. In the legend SC5-18-iv refers to the semicarbazide mPEG5000 modified EPO with 18 molecules of mPEG /molecule of EPO and injected intravenously, EPO-iv refers to injected intravenously.

Figure 8 is a graph showing changes in hematocrit level in response to injection with EPO. In the legend, the term SC5-18 refers to the semicarbazide mPEG5000 modified EPO with 18 molecules of mPEG /molecule of EPO, the term SC5-22 refers to the semicarbazide mPEG5000 modified EPO with 22 molecules of mPEG5000 /molecule of EPO, the term HY5-

8 refers to the hydrazide mPEG5000 modified EPO with 8 molecules of mPEG5000 /molecule of EPO.

Figure 9 is a graph showing changes in hematocrit level in response to injection with EPO. In the legend, the term SC5-24 refers to the semicarbazide mPEG5000 modified EPO with 24 molecules of mPEG /molecule of EPO, the term TS5-25 refers to the thiosemicarbazide mPEG5000 modified EPO with 25 molecules of mPEG5000 /molecule of EPO, the term DH5-22 refers to the dihydrazide mPEG5000 modified EPO with 22 molecules of mPEG5000 /molecule of EPO, M.A. refers to the mouse albumin control.

Figure 10 is a graph showing changes in hematocrit level in response to injection with EPO. In the legend, the term TS5-17 refers to the thiosemicarbazide mPEG5000 modified EPO with 17 molecules of mPEG5000 /molecule of EPO, the term SC8.5-12 refers to the semihydrazide mPEG8500 modified EPO modified with 12 molecules of mPEG8500 /molecule of EPO, SC2-15 refers to the semicarbazide mPEG2000 modified EPO with 15 molecules of mPEG2000 /molecule of EPO, M.A. refers to the mouse albumin control.

Figure 11 is a graph showing changes in hematocrit level in response to injection with EPO. The legend is as follows: the term SC5-18 refers to the mPEG5000 semicarbazide modified EPO with 18 molecules of mPEG5000 /molecule of EPO, the term SC12-14 refers to the semicarbazide mPEG12,000 modified EPO with 14 molecules of mPEG12,000 /molecule of EPO, the term SC5-28 refers to the semihydrazide mPEG5000 modified EPO with 28 molecules of mPEG5000 /molecule of EPO, the term HY12-6 refers to the hydrazide mPEG12,000 modified EPO with 6

molecules of mPEG12,000 /molecule of EPO, M.A. refers to the mouse albumin control.

Figure 12 is a graph showing changes in hematocrit level in response to injection with EPO. In the legend, the term SC8.5-34 refers to the semicarbazide mPEG8500 modified EPO with 34 molecules of mPEG8500 /molecule of EPO, the term SC12-29 refers to the semicarbazide mPEG12,000 modified EPO with 29 molecules of mPEG12,000 /molecule of EPO, M.A. refers to the mouse albumin control.

Figure 13 is a graph of hematocrit levels in mice injected subcutaneously or intravenously EPO and EPO derivatives. The legend is as follows: EPO-SC refers to EPO injected subcutaneously, EPO-iv refers to EPO injected intravenously, SC5-28-SC refers to semicarbazide mPEG5000 modified EPO with 28 molecules of mPEG5000 /molecule of EPO injected subcutaneously, SC5-28-iv refers to semicarbazide mPEG5000 modified EPO with 28 molecules of mPEG5000 /molecule of EPO injected intravenously, HY12-6-SC refers to hydrazide mPEG12,000 modified EPO with 6 molecules of mPEG12,000 /molecule of EPO injected subcutaneously, HY12-6-iv refers to hydrazide mPEG12,000 modified EPO with 6 molecules of mPEG12,000 /molecule of EPO injected intravenously, M.A.-sc refers to mouse albumin control injected subcutaneously, M.A.-iv refers to mouse albumin control injected intravenously.

Figure 14 is a graph showing hematocrit levels in mice injected with multiple versus single doses of .1 micrograms of EPO. The legend is as follows: EPOx3sc refers to EPO injected subcutaneously three times a week, EPOx3iv refers to EPO injected intravenously three times a week, SC5-22x1sc refers

to semicarbazide mPEG5000 modified EPO with 22 molecules of mPEG5000 /molecule of EPO injected subcutaneously once a week, SG5-22x1iv refers to semicarbazide mPEG5000 modified EPO with 22 molecules of mPEG5000 /molecule of EPO injected intravenously once a week, M.A.x3 refers to control mouse albumin injected intravenously three times a week.

Figure 15 is a graph showing hematocrit levels in mice with tumor necrosis factor α(TNFα)-induced anemia and injected with EPO and derivatives of EPO. The legend is as follows: TNF(5) refers to TNF injected over five days, T+EPO(5) refers to TNF and EPO injected simultaneously over a period of five days, T+EPO(2) refers to TNF injected over a period of five days and EPO injected on days 1 and 4, T+SC5-18(5) refers to TNF injected over a period of five days simultaneously with semicarbazide mPEG5000 modified EPO with 18 molecules of mPEG5000 /molecule of EPO, T+SC5-18(2) refers to TNF injected over a period of five days simultaneously with semicarbazide mPEG5000 modified EPO with 18 molecules of mPEG5000 /molecule of EPO injected on days 1 and 4, M-A refers to the mouse albumin control.

Figure 16 is a graph showing hematocrit changes in response to injection with EPO. In the legend, 18PEG-A refers to EPO modified with mPEG-O-$CH_2CH_2$-NH-CO-$ONH_2$, (formula XXI of the invention), with 18 mPEG molecules per molecule of EPO; 31 PEG-C refers to EPO modified with mPEG-O-$CH_2CH_2$-NH-CO-$CH_2$-$ONH_2$ (formula XXIV of the invention), with 31 mPEG/EPO; 25PEG-C refers to EPO modified with mPEG-O-$CH_2$-$CH_2$-NH-CO-$CH_2$-$ONH_2$ (formula XXIV of the invention), with 25 molecules of mPEG/molecule EPO.

Figure 17 is a graph showing hematocrit changes in response to injection with EPO. In the legend, 22 PEG-B refers to EPO modified with the oxime-derivatized mPEG-O-$CH_2CH_2$-$ONH_2$ (formula XXIII of the invention), with 22 mPEG molecules/molecule of EPO; 17 PEG-B refers to EPO modified with the same oxime linker having 17 mPEG molecules/molecule of EPO, and 12 PEG-B refers to EPO modified with the same oxime linker having 12 mPEG molecules/molecule of EPO.

Figure 18 is a graph showing the ability of EPO, m-PEG-O-$CH_2CH_2$-NH-CO-$ONH_2$ ("A") mPEG5000 EPO (18 PEG/EPO), mPEG-O-$CH_2CH_2$-$ONH_2$ ("B") mPEG5000 EPO (22 PEG/EPO), mPEG-O-$CH_2CH_2$-$ONH_2$("B") mPEG5000 EPO (17 PEG/EPO),mPEG-O-$CH_2CH_2$-$ONH_2$ ("B") mPEG5000 EPO (12 PEG/EPO), mPEG-O-$CH_2CH_2$-NH-CO-$CH_2$-$ONH_2$ ("C") mPEG5000 EPO (31 PEG/EPO), and mPEG-O-$CH_2CH_2$-NH-CO-$CH_2$-$ONH_2$ ("C") mPEG5000 EPO (25 PEG/EPO) to bind a monoclonal antibody specific for EPO in an ELISA assay.

Figure 19 is a graph showing EPO dependent cell proliferation using mPEG-EPOs. In the legend 18 PEG-A refers to EPO modified with mPEG-O-$CH_2CH_2$-NH-CO-$ONH_2$ (formula XXI) at 18 PEG/EPO, 22 PEG-B refers to EPO modified with mPEG-O-$CH_2CH_2$-$ONH_2$ (formula XXIII) at 22 PEG/EPO, 17 PEG-B refers to formula XXIII at 17 PEG/EPO, 12 PEG-B refers to formula XXIII at 12 PEG/EPO, 31 PEG-C refers to EPO modified with mPEG-O-$CH_2CH_2$-NH-CO-$CH_2$-$ONH_2$ (formula XXIV) at 31 PEG/EPO, and 25 PEG-C refers to formula XXIV at 25 PEG/EPO.

Figure 20 is a graph showing hematocrit changes in response to injection with EPO. In the legend, 31 mPEGs refers to EPO modified with the oxime-derivatized mPEG-O-CO-$NHNH_2$ (formula II of the invention), having 31 mPEG molecules/molecule EPO.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The term "water-soluble polymer reagent" as used herein, refers to a water-soluble polymer modified so as to contain a functional group that provides for the covalent conjugation of the water-soluble polymer to a polypeptide.

The term "polypeptide" as used herein, refers to polypeptides of various sizes, including larger polypeptides (frequently referred to as proteins), small peptides, and glycoproteins.

The term "oxidation activatable group" as used herein, refers to functional groups such as alcohols, polyols, lactols, amines, phenols, carboxylic acids, or carboxylic acid derivatives that react with the hydrazide portion or the oxylamine portion of the subject compounds after the functional group has been exposed to oxidative conditions. Oxidation activatable groups present on a polypeptide that is a glycoprotein may be present on the carbohydrate portion of the glycoprotein or on the amino acid residue portion of the glycoprotein. Exemplary, but not exclusive, of oxidation activatable groups are hydroxyl groups present on the carbohydrate portion of glycoproteins. The hydroxyl groups may be oxidized to hydrazide reactive aldehydes or oxylamine reactive aldehydes, depending on the derivative employed.

The term "partial oxidation" as used herein, refers to the processes of oxidation that proceed to an extent that does not completely abolish the biological activity of the polypeptide being oxidized.

The term "activated for conjugation" as used herein with respect to polypeptides, refers to the

partial oxidation of a polypeptide, where the extent of oxidation is sufficient to convert at least one oxidation activatable group to a functional group capable of chemically reacting with the hydrazide portion or oxylamine portion (or similar functional group portion) of one of the subject water-soluble polymer reagents.

The term "biological activity" as used herein, refers to biologically relevant properties of a compound including: enzymatic activity, the ability to bind to receptors (including antibodies), the ability to bind ligands, the ability to induce an immune response, therapeutic activity and the like.

The term "antibodies," as used herein, includes both polyclonal and monoclonal antibodies with natural immunoglobulin sequences, synthetic antibody derivatives, and the like; antibodies may be modified so as to be joined to any of a variety of labels, fluorescent, radioactive, enzymatic, biotin/avidin or the like. Synthetic antibody derivatives include natural immunoglobulin sequences that have been mutated and selected for altered binding specificity, various immunoglobulin gene derived polypeptides, typically single chain, produced by genetically modified bacteria, antibodies modified so as to contain modified constant regions and the like; a review of such synthetic antibody derivatives based on the principles of antibody formation is provided in Winter and Milstein, Nature, 349: 293-299 (1991).

The Invention

The subject invention provides novel polypeptide modifying reagents that are hydrazine or oxylamine derivatives of water-soluble polymers such as PEG,

i.e, polyethylene glycol, for use in modifying polypeptides so as to be bound to water-soluble polymers. The water-soluble polymer reagents of the subject invention may be used to covalently attach a variety of water-soluble polymers to polypeptides of interest. The subject hydrazine and oxylamine derivatives of water-soluble polymers, i.e., water-soluble polymer reagents, may be covalently attached to proteins through reactions with aldehyde groups or other suitable functional groups present on the protein of interest. Aldehyde groups may be introduced by partially oxidizing the hydroxyl groups (or other oxidation activatable groups) on the polypeptide. Examples of oxidation activable groups include the hydroxyl groups present on the carbohydrate moieties of a glycoprotein. Suitable methods of oxidation, i.e., partial oxidation, include treating the polypeptide of interest with an oxidizing agent such as periodate or other oxidation agents known to those of skill in the art, or adding an enzyme capable of catalyzing oxidation reactions on portions of the protein of interest, e.g., galactose oxidase. Another aspect of the subject invention is to provide polypeptides modified by the reagent molecules, i.e., the subject water-soluble polymer hydrazine or oxylamine derivatives, so as to be covalently bonded to one or more water-soluble polypeptides.

Preferred formulae of the compounds useful for coupling water-soluble polymers to polypeptides are as follows:

HYDRAZINE DERIVATIVES

(I) $P-O-CH_2-CO-NHNH_2$,

a hydrazine derivative;

(II) P-O-CO-NHNH$_2$,
a hydrazine carboxylate derivative;

(III) P-NH-CO-NHNH$_2$,
a semicarbazide derivative;

(IV) P-NH-CS-NHNH$_2$,
a thiosemicarbazide derivative;

(V) P-NHCO-NHNHCO-NHNH$_2$,
a carbonic acid dihydrazide derivative;

(VI) P-NHNHCONHNH$_2$,
a carbazide derivative;

(VII) P-NHNHCSNHNH$_2$,
a thiocarbazide derivative;

(VIII) P-NH-CO-C$_6$H$_4$-NHNH$_2$,
an aryl hydrazide derivative;

(IX) P-O-CO-CH$_2$CH$_2$-CO-NHNH$_2$,
a hydrazide derivative;

OXYLAMINE DERIVATIVES

(XIX) P-O-CH$_2$CH$_2$-CO-ONH$_2$;

(XX) P-O-CH$_2$CH$_2$-O-CO-ONH$_2$;

(XXI) P-O-CH$_2$CH$_2$-NH-CO-ONH$_2$;

(XXII) P-O-$CH_2CH_2$-NH-CS-$ONH_2$;

(XXIII) P-O-$CH_2CH_2$-$ONH_2$;

(XXIV) P-O-$CH_2CH_2$-NH-CO-$CH_2ONH_2$;

(XXV) P-O-$CH_2CH_2$-O-CO-$CH_2$-$ONH_2$;

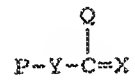
(XXVI) P-O-$CH_2CH_2$-CH(OH)-$CH_2$-$ONH_2$; and

(XXVII) P-O-$CH_2CH_2$-CO-$CH_2$-$ONH_2$.

P represents a water-soluble organic polymer in the above formulae. Water-soluble organic polymers of interest have hydroxyl groups appended to the polymer backbone and may be selected from known water-soluble polymers including but not limited to: (a) dextran and dextran derivatives, including dextran sulfate, P-amino cross linked dextrin, and carboxymethyl dextrin (b) cellulose and cellulose derivatives, including methylcellulose and carboxymethyl cellulose (c) starch and dextrines, and derivatives and hydroylactes of starch (d) polyalklyene glycol and derivatives thereof, including polyethylene glycol, methoxypolyethylene glycol, polyethylene glycol homopolymers, polypropylene glycol homopolymers, copolymers of ethylene glycol with propylene glycol, wherein said homopolymers and copolymers are unsubstituted or substituted at one end with an alkyl group (e) heparin and fragments of heparin, (f) polyvinyl alcohol and polyvinyl ethyl ethers, (g) polyvinylpyrrolidone, (h) α,β-Poly[(2-hydroxyethyl)-DL-aspartamide, and (i) polyoxyethylated polyols.

Preferably, the water-soluble polymer P is selected from dextran and dextran derivatives, dextrine and dextrine derivatives, and more preferably polyethylene glycol and derivatives thereof. Polyethylene glycol water-soluble polymers include polyethylene glycol where one of the terminal hydroxyl group is modified with an R group, i.e., RO-PEG, where R may be alkyl, aryl, alkyaryl, aroyl, alkanoyl, benzoyl, arylalkylethers, cycloalkyl, cycloalkylaryl, and the like. The water-soluble polymers listed are only exemplary of water-soluble polymers represented by P. Various derivatives of the specifically recited water-soluble polymers are also contemplated, provided that the derivatives are water-soluble. More preferably, the water-soluble polymer P is selected from the group consisting of polyethylene glycol and derivatives thereof, the monomethyl ether of polyethylene glycol (mPEG) being particularly preferred (so as to avoid cross-linking between proteins). When polypeptides modified by the water-soluble polymer reagents of the subject invention are to be used as pharmaceuticals, polymer P should be non-toxic.

The compounds of formulae I-IX may be represented generally by the formula:



wherein X is O or S; Q is selected from the group consisting of $-NHNH_2$, and $-C_6H_4-NHNH_2$; and Y is selected from the group consisting of -O-, $-OCH_2-$, -NH-, -NHNH-, $-O-CO-CH_2CH_2-$ and -NHCO-N-NHNH-; and P is a water- soluble organic polymer (as in compounds I-IX).

The compounds of formulae XIX-XXVII may be represented generally by the formula:

P-Y-X-Q

wherein X is C=O, C=S, $CH_2$ or CHOH; Q is selected from the group consisting of $-ONH_2-$, and $-CH_2-ONH_2-$, and Y is selected from the group consisting of $-O-CH_2CH_2-$, $-O-CH_2CH_2-O-$, $-O-CH_2CH_2-N-$, $O-CH_2CH_2-S$, and $-O-CH_2CH_2CH-$; and P is a water soluble organic polymer (as in compounds XIX-XXVII).

In addition to the molecules of formulae I, II, III, IV, V, VI, VII, VIII, and IX the subject invention also includes polypeptides modified by reaction with the molecules of formulae I, II, III, IV, V, VI, VII, VIII, and IX. Polypeptides modified by the water-soluble polymer reagents of formulae I, II, III, IV, VI, VII, VIII, and IX may be represented by formulae X, XI, XII, XIII, XIV, XV, XVI, XVII, and XVIII, respectively:

HYDRAZIDE-MODIFIED POLYPEPTIDES

(X) $[P-O-CH_2-CO-NHN=CH-]_n-Z$,

(XI) $[P-O-CO-NHN=CH-]_n-Z$,

(XII) $[P-NH-CO-NHN=CH-]_n-Z$,

(XIII) $[P-NH-CS-NHN=CH-]_n-Z$,

(XIV) $[P-NHCO-N-NHNHCO-NHN=CH-]_n-Z$,

(XV) $[P-NHNCON=CH-]_n-Z$,

(XVI) $[P-NHNCSN=CH-]_n-Z$,

(XVII) $[P-NH-CO-C_6H_4-NHN=CH-]_n-Z$, and

(XVIII) $[P-O-CO-CH_2CH_2-CO-NHN=CH-]_n-Z$,

wherein P is a water-soluble polymer as previously described, Z represents a polypeptide, as described above, and n represents a number in the range 1 to x, where x is the maximum number of oxidation activatable groups present in polypeptide Z. The C in the hydrazone linkage formed between the water-soluble polymer reagent and Z was originally present on Z, not the water-soluble polymer reagent.

In addition to the molecules of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII the subject invention also includes polypeptides modified by reaction with the molecules of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII. Polypeptides modified by the water-soluble polymer reagents of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII may be represented by formulae XXVIII, XXIX, XXX, XXXI, XXXII, XXXIII, XXXIV, XXXV and XXXVI, respectively:

OXYLAMINE-MODIFIED POLYPEPTIDES

(XXVIII) $[P-O-CH_2CH_2-CO-ON=CH-]_n-Z$;

(XXIX) $[P-O-CH_2CH_2-O-CO-ON=CH-]_n-Z$;

(XXX) $[P-O-CH_2CH_2-NH-CO-ON=CH-]_n-Z$;

(XXXI) $[P-O-CH_2CH_2-NH-CS-ON=CH-]_n-Z$;

(XXXII) $[P-O-CH_2CH_2-ON=CH-]_n-Z$;

(XXXIII) $[P-O-CH_2CH_2-NH-CO-CH_2-ON=CH-]_n-Z$;

(XXXIV) $[P-O-CH_2CH_2-O-CO-CH_2-ON=CH-]_n-Z$;

(XXXV) $[P-O-CH_2CH_2-CH(OH)-CH_2-ON=CH-]_n-Z$; and

(XXXVI) $[P-O-CH_2CH_2-CO-CH_2-ON=CH-]_n-Z$,

wherein P is a water-soluble polymer as previously described, Z represents a polypeptide, as described above, and n represents a number in the range 1 to X, where X is the maximum number of oxidation and activatable groups present in polypeptide Z. The carbon atom in the oxime linkage formed between the water-soluble polymer reagent and Z was originally present on Z, not the water-soluble polymer reagent.

Although polypeptides may be modified by the coupling of up to x water-soluble polymers per polypeptide molecule, it may be desirable to modify a given polypeptide by less than x water-soluble polymer molecules. It may be undesirable to derivatize a polypeptide with the maximum number of water-soluble polymers, i.e., x water-soluble polymers/polypeptide molecule, because for some polypeptides, increasing the number of water-soluble polymers per molecule of polypeptide may diminish biological activities as compared the unmodified polypeptide. For example see figures 2, 4, 5, 9, 10,

and 11, for some results obtained with water-soluble polymer modified EPO.

Different methods of measuring the number of water-soluble polymer molecules attached to a glycoprotein molecule, as in hydrazone linked compounds of formulae X, XI, XII, XIII, XIV, XV, XVI, XVII, and XVIII and as in oxime linked compounds of formulae XXVIII, XXIX, XXX, XXXI, XXXII, XXXIII, XXXIV, XXXV and XXXVI may give different results. For the purpose of this application, when a polypeptide is said to be derivatized by a given number of water-soluble polymer molecules/molecule of protein, the number of water-soluble polymers given is the empirically determined figure measured by gel filtration chromatography retention time.

The synthesis of compounds of formulae X, XI, XII, XIII, XIV, XV, XVI, XVII, and XVIII may result in the creation of a mixture of reaction products differing from one another with respect to the exact number of water-soluble polymers attached to the polypeptide through hydrazone linkages and the sites on the polypeptide where these hydrazone linkages are present. Similarly, the synthesis of compounds of formulae XXVIII, XXIX, XXX, XXXI, XXXII, XXXIII, XXXIV, XXXV and XXXVI may result in the creation of a mixture of reaction products differing from one another with respect to the exact number of water-soluble polymers attached to the polypeptide through oxime linkage and the sites on the polypeptide where these oxime linkages are present. As the polymer P comprises multiple identical units of varying amounts, it will be appreciated that the molecular weight of P may vary considerably. Furthermore, when P is said to have a given molecular weight, that

molecular weight may only be approximate, reflecting the average molecular weight of a population of molecules P differing with respect to one another in regards to the number of subunits present in the molecule. In general, P will have a molecular weight of about 200 to 200,000, preferably in the range of 700 to 30,000, more preferably in the range of 2,000-12,000. Suitable molecular weights for P, when the molecules of formulae I, II, III, IV, V, VI, VII, VIII, and IX and the molecules of formulae XXVIII, XXIX, XXX, XXXI, XXXII, XXXIII, XXXIV, XXXV and XXXVI are to be coupled to a polypeptide will vary in accordance with the specific polypeptide to be modified and the specific water-soluble polymer selected. Individual polypeptide molecules may be derivatized by one or more different water-soluble polymers by means of reaction with different embodiments of the compounds of formulae I, II, III, IV, V, VI, VII, VIII, and IX (the hydrazones), or the compounds of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII (the oximes), or any combination of the hydrazones and the oximes.

An advantage of the subject invention is that polypeptides may be modified by the attachment of water-soluble polymers without substantially reducing the biological activity of the polypeptide, or reducing the biological activity to a lesser extent than the biological activity would be reduced by the attachment of a similar number of the same water-soluble polymers/polypeptide molecule by means of previously known chemical coupling methods and compounds. Aspects of the biological activity of EPO include the stimulation of red blood cell formation. A detailed description of the biological activity of

EPO can be found in Krantz, S.B., Blood 77: 419-434 (1991).

Another advantage of the subject invention is that polypeptides modified by the compounds of formulae I, II, III, IV, V, VI, VII, VIII, and IX or the compounds of formulae XIX, XX, XXI, XXII, XXIII, XXIV, XXV, XXVI and XXVII may then retain a greater degree of their biological activity than when the same polypeptide is modified to the same degree by joining water-soluble polymers to polypeptides employing the frequently used (prior to the subject invention) active esters of mPEG for lysine modification. Thus, the subject invention provides for modified polypeptides that possess the advantages associated with the covalent conjugation of water-soluble polymers while minimizing the loss of biological activity associated with the modification. Consequently, polypeptides that may be more highly derivatized by water-soluble polymers, and thus or otherwise possess the advantages associated with the higher degree of derivatization, may be produced that have the same level or a higher level of biological activity as polypeptides derivatized by water-soluble polymers to a lesser extent using conventional methodology.

Another advantage of using a hydrazone forming derivatives of PEG (and other water-soluble polymers) instead of PEG-amine for coupling PEG to a protein is that coupling of a hydrazide or oxylamine (or similar compounds) to an aldehyde yields a hydrazone or oxime respectively, while coupling through an amine gives an imine, which is less stable than a hydrazone or an oxime and needs to be reduced to give a stable derivative. Thus an extra step is required when

using an amine instead of a hydrazone forming compound.

Another advantage of the subject invention is that higher levels of water-soluble polymers may be attached to glycoproteins than with other water-soluble polymer derivatives. The semicarbazide (formula III), thiosemicarbazide (formula IV), and carbonic acid dihydrazide (formula V) derivatives are of particular interest because of their higher reactivity than comparable hydrazide derivatives of the subject invention. Reactions involving the mPEG derivatization of EPO with semicarbazides, thiosemicarbazides and carboxylate hydrazide described herein have resulted the addition of up to about 31-34 mPEG molecules for each molecule of EPO, whereas similar reactions using corresponding hydrazide derivatives of EPO have resulted in the addition of about 6-12 molecules of mPEG to each molecule of EPO. Reactions between carbonic acid dihydrazide and hydrazide carboxylate derivatives and EPO have resulted in the addition of up to 22 mPEG molecules to each molecule of EPO. In order for hydrazide derivatives of mPEG to incorporate about 20 mPEG molecules to each molecules of EPO, very strong oxidation conditions were required, e.g., 50 mM periodate, 60 minutes incubation at room temperature. The subject mPEG semicarbazide and thiosemicarbazide derivatives could be used to provide EPO modified with PEG to a similar extent, but under more mild oxidation conditions, e.g., 10 mM periodate, for 5-15 minutes at 0°C. Strong oxidizing conditions may have an adverse effect on the structural and biological properties of many polypeptides, thus PEG semicarbazide, carbonic acid dihydrazide, hydrazide

carboxylate, and thiosemicarbazide derivatives may be particularly useful compounds for modifying polypeptides with PEG (or other water-soluble polymers).

A novel series of oxylamine derivatives of mPEG have been synthesized and have been reacted to the oxidized carbohydrate groups of EPO. Some of the mPEG-oxylamines showed high reactivity to the oxidized carbohydrates. Also a lower number of mPEGs could be incorporated onto EPO and still give the high in vivo activity as seen with the semicarbazide and carboxylate hydrazide (hydrazone forming) mPEG-derivatives. This lower number for mPEG incorporation is advantageous in that shorter and milder oxidation conditions can be used in the modification. Also lesser amounts of mPEG-derivative can be used in the modification reaction.

Similarly, particularly high levels of water-soluble polymers are attached to glycoproteins when the compounds of formula XXI, formula XXIV, and formula XXIII are employed as compared to comparable formula XIX oxylamine derivative. Reactions involving derivatization of EPO with formula XXI and formula XXIV described herein have resulted in the addition of up to 18-19 mPEGS/EPO, and 31 mPEG molecules for every molecule of EPO, respectively, whereas similar reactions using corresponding formula XXII and XIX oxylamine derivatives of EPO have resulted in the addition of about 3-4 molecules of mPEG to each molecule of EPO. Perhaps more importantly, the bioactivity of resulting oxylamine-derivatized PEG-EPO is surprisingly high even at more moderate levels of attachment of water-soluble polymer to EPO (see hereinbelow and Figure 17). In

this regard, bioactivity of a 12 mPEG isolated fraction of formula XXIII is of particular interest (See Figures 16 & 17).

The ability to generate long acting mPEG-EPO with high activity via coupling mPEG to the oxidized carbohydrate groups depends on the mPEG-derivative chosen. Some mPEG carbohydrate modifying derivatives are not reactive enough to attach an optimum amount of mPEG onto EPO. Some mPEG-derivatives require a high amount of incorporation onto EPO due to the stability of the resulting bond. An optimal amount of mPEG incorporation for the semicarbazide derivative is about 17-25, more preferably about 22; for the carboxylate hydrazide derivative, about 22-32, more preferably about 31. Reactivity of the mPEG-oxylamines is about 3-36 mPEGs/EPO.

The water-soluble polymer reagents of the subject invention may be used to modify a variety of polypeptides or similar molecules that contain aldehydes or functional groups with similar chemical reactivity, e.g., ketones, lactols, activated carboxylic acids or activated carboxylic acid derivatives, capable of chemically reacting with the hydrazide portion (or similar functional portion) of the subject water-soluble polymer reagent derived from the oxidation of hydroxyl groups, the oxidation of other oxidation activatable groups present on the polypeptide of interest (including carbohydrate moieties when the polypeptide is a glycoprotein, and amino acid residues in the primary sequence, e.g., the N-terminus of serine, threonine, hydroxylysines), or hydrazine or oxylamine reactive group present on polypeptides prior to or after any oxidative

treatment. Polypeptides of interest include antibodies, monoclonal and polyclonal, cytokines, growth factors, hormones, enzymes, protein or peptide ligands and the like. Polypeptides of interest for modification by hydrazone linkage or oxime linkage forming water-soluble polymer reagent molecules of the subject invention may be isolated from their natural sources, genetically engineered cells, e.g., CHO cells transformed with expression vectors for the production of EPO, or produced by various in vitro synthesis methods. A particularly preferred polypeptide for the purposes of the instant invention is EPO, and precursors, intermediates and mimetics thereof, whether human or recombinant.

While the water-soluble polymer reagents of the subject invention may be used to modify most polypeptides, it is of particular interest to modify (1) polypeptides for use as drugs, and (2) polypeptides for use in assays. Polypeptide for use in assays include specific binding proteins, polypeptides recognized by specific-binding proteins, and enzymes. By specific-binding proteins it is intended antibodies, hormone receptors, lectins, and the like.

Various polypeptides may be modified by the subject water-soluble polymer reagents and the subject methods for their use so as to be coupled to different water-soluble polymers and to differing degrees or modification. Varying parameters such as (1) the number of water-soluble polymers coupled to an individual polypeptide molecule, which will depend upon the reactivity of the derivatized mPEGs to the EPO, and the bioactivity of the resulting mPEG-EPO; e.g., reactivity from about 3-36 molecules of

mPEG/EPO (2) the molecular weight of the water-soluble polymer, e.g., 2,000-12,000 daltons (3) the structure of the water-soluble polymer, e.g., monomethoxypoly(ethylene glycol) (4) the reaction conditions under which the reaction between the water-soluble polymer reagent and the polypeptide of interest, e.g., temperature and duration, and (5) the oxidation conditions under which the polypeptide for modification is activated for covalent conjugation, e.g., periodate at a concentration in the range of 10-40 μmol/mg of protein, may influence the biological properties of the resultant water-soluble polymer modified polypeptide.

In a preferred embodiment of the invention, activation of polypeptides for covalent conjugation is performed by mixing the protein for modification with periodate (0.1-1,000 μmole/mg protein) for a period of time in the range of one minute to three days, more preferably 0.5-50 μmole periodate/mg protein, for a time period in the range of 5 minutes to 180 minutes. In a preferred embodiment of the invention, activation for conjugation is performed by mixing the protein for modification with periodate at a temperature in the range of -10°-50°C, more preferably in the range of 0°-30°C.

In a preferred embodiment of the subject invention when the protein for modification is EPO, EPO is derivatized with the compounds of formulae II-VIII, more preferably the compounds of formulae II-V, the compound of formula III, the semicarbazide, and formula II, the carboxylate hydrazide, being particularly preferred, where the water-soluble polymer P is methoxypolyethylene glycol (mPEG) and each molecule of EPO is derivatized by 3-36, more